

## FERRITIN IN THE FUNGUS *PHYCOMYCES*

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### ABSTRACT

The iron-protein ferritin has been purified from mycelium, sporangiophores, and spores of the fungus *Phycomyces blakesleeanus*. It has a protein-to-iron ratio of 5, a sedimentation coefficient of 55S, a buoyant density in CsCl of 1.82 g/cm<sup>3</sup>, and the characteristic morphology of ferritin in the electron microscope. Apoferritin prepared from *Phycomyces* ferritin has a sedimentation coefficient of 18S and consists of subunits of molecular weight 25,000. In the cytoplasm of *Phycomyces*, ferritin is located on the surface of lipid droplets (0.5–2.0  $\mu$  in diameter) where it forms crystalline monolayers which are conspicuous in electron micrographs of sporangiophore thin-sections. Ferritin is found in all developmental stages of *Phycomyces* but is concentrated in spores. The level of ferritin iron is regulated by the iron level in the growth medium, a 50-fold increase occurring on iron-supplemented medium.

### INTRODUCTION

Ferritin is a large protein containing up to 30% iron by weight. It was discovered in horse spleen and first purified by Laufberger (1937). Since then, ferritin from mammalian tissues has been extensively studied (see Granick, 1946, and Harrison, 1964, for reviews). More recently, ferritin has been demonstrated in invertebrates (Roche et al., 1961; Towe et al., 1963) and plants (Hyde et al., 1963). This paper presents evidence that it is also present in the fungus *Phycomyces blakesleeanus*, indicating an even wider occurrence of this protein than was previously supposed.

Ferritin consists of a core of ferric hydroxide surrounded by a spherical protein shell (apoferritin) which is made up of 20 identical subunits, each of which has a molecular weight of 24,000 (Hofmann and Harrison, 1963). The iron core is sufficiently electron-opaque to be visible in the electron microscope in unstained preparations, while apoferritin appears in negatively stained preparations as an electron-lucent an-

nulus around the core (Richter, 1959). Due to its iron content, ferritin has a high buoyant density in CsCl (1.8 g/cm<sup>3</sup>) and a high sedimentation coefficient (65–70S). Moreover, the iron content of individual molecules can vary from no iron (apoferritin) to about 4000 atoms in a full core (Fischbach and Anderegg, 1965). Ferritin molecules with different iron contents have correspondingly different sedimentation coefficients and buoyant densities. In ferritin preparations from mammalian liver and spleen, a heterogeneous distribution of iron contents and apoferritin (up to 25%) are observed.

The first part of this paper describes the purification and properties of *Phycomyces* ferritin and the extent to which it is different from mammalian (e.g. horse) ferritin. The second part describes the unique localization of *Phycomyces* ferritin on the surface of lipid droplets in cytoplasm (Sassan, 1965; Peat and Banbury, 1968) and its distribution in the various developmental stages—

mycelium, sporangiophores, and spores. Because of the availability of these distinct developmental stages, *Phycomyces* is a very suitable organism for studying the role of ferritin in the metabolism, transport, and storage of iron. Results are also presented which demonstrate that ferritin synthesis in *Phycomyces* is regulated by iron concentration in the growth medium. Further studies on *Phycomyces* ferritin will be published elsewhere.

## MATERIALS AND METHODS

### *Stocks of Phycomyces blakesleeana*

Experiments were done with mycelium, vegetative spph's,<sup>1</sup> and spores. The strain used was NRRL 1555(—), which has been used for biophysical experiments (see review by Bergman et al., 1969).

Mycelium was prepared by germinating spores in aerated liquid culture. After 2–5 days of growth (at 20–23°C), the mycelium was harvested by filtration through a Buchner funnel. To prepare spph's, spores were germinated on solid medium in Petri dishes. After 3 days, mycelial growth covered the agar surface and spph's were initiated. On GA medium, about 3,000 spph's grew on a single Petri dish. Spph's (4–6 cm high) with a mature sporangium were harvested by cutting them off with scissors or plucking them from the mycelium.

### *Media*

A synthetic glucose-asparagine (GA) medium was used for all experiments (Bergman et al., 1969). The medium was solidified by the addition of 1% agar.

To induce higher levels of ferritin biosynthesis, 15 µg/ml Fe (as FeCl<sub>3</sub>) was added to liquid cultures; 500 µg Fe/Petri dish was added to the solid medium.

### *Reagents*

Horse ferritin (2× crystallized) and tripyridyl-s-triazine were obtained from Sigma Chemical Co., St. Louis, Mo. Bovine gamma globulin was obtained from Behringwerke AG, Marburg-Lahn, Germany. <sup>59</sup>FeCl<sub>3</sub> in 0.1 M HCl (5.8 mCi/mg) was obtained from New England Nuclear Corp., Boston, Mass.

### *Electron Microscopy*

*Phycomyces* spph's were fixed for 2 hr in 2.5% glutaraldehyde at room temperature, followed by osmium tetroxide at 4°C for ½ hr. Specimens were dehydrated with ethanol and propylene oxide prior

to embedding in Maraglas (The Marblette Co. Div. of Allied Products Corp., Long Island City, N. Y.). Thin sections were cut on an LKB microtome and stained briefly with 0.5% lead citrate.

Purified ferritin was prepared for examination by placing a drop of ferritin solution onto a grid covered with a thin film of carbon. After allowing a minute for adsorption, the excess was removed by a thorough washing with distilled water and the sample was allowed to dry. Negative staining was achieved by adding a drop of a saturated aqueous solution of uranyl acetate to the grid before drying. After 15 sec. this solution was diluted with an equal volume of water, and then the liquid on the grid was absorbed with filter paper.

Unstained material was examined in a Phillips EM 200 operating at 40 kv with a 20 µ aperture; stained material was examined at 80 kv with a 40 µ aperture.

Electron diffraction was performed on unstained films of ferritin by the selected area method in a Siemens Elmiskop I with a 200 µ aperture (Alderson and Halliday, 1965).

### *Ultracentrifugation*

Preparative centrifugations were carried out with SW-39 and SW-50 rotors in a Spinco Model L ultracentrifuge (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.). Analytical runs for sedimentation velocity and buoyant density determinations were performed in a Spinco Model E ultracentrifuge with UV absorption optics. The resulting photographs were traced with a Joyce-Loebl (Joyce, Loebl and Co., Gateshead-on-Tyne, England) microdensitometer.

### *Acrylamide Gel Electrophoresis*

5% gels (90 × 6 mm) were made by combining: two volumes of 0.2 M sodium phosphate buffer, pH 7.5, two volumes of 20% acrylamide (0.53% methylenebisacrylamide), three volumes of water, one volume of 0.56% ammonium persulfate (freshly made), and 20 µl *N,N,N*-tetramethylethylenediamine per 100 ml gel solution. The electrode buffer was 0.05 M sodium phosphate, pH 7.5. Samples (50–100 µl) were applied in 10% glycerol. Electrophoresis was carried out for about 12 hr at 5 mamp/gel.

Sodium dodecylsulfate (SDS)-acrylamide gel electrophoresis was performed in a continuous buffer system of 0.1 M sodium phosphate, pH 7.0, 0.1% in SDS, according to the procedure of Weber and Osborn (1969).

Gels were stained with Coomassie Brilliant Blue and destained electrophoretically (Weber and Osborn, 1969).

Dissociation of ferritin with SDS was performed by incubating 500 µg samples for 2 hr at 37°C in a

<sup>1</sup> Sporangiophore is abbreviated spph throughout this paper.

solution of 1% SDS, 1% mercaptoethanol, and 0.1 M sodium phosphate buffer, pH 7.0, followed by dialysis overnight in a solution of 0.1% SDS, 0.1% mercaptoethanol, and 0.01 M sodium phosphate, pH 7.0.

### *Determination of Protein and Iron*

#### *Content of Ferritin*

The amounts of protein and iron present were determined in hydrolysates of ferritin with ninhydrin reagent (for amino acids) and tripyridyl-*s*-triazine (for iron). Ferritin samples were hydrolyzed in 6 N HCl (approximately 500 µg/ml) at 110°C for 20 hr in sealed tubes under nitrogen. After hydrolysis, samples were evaporated to remove HCl and taken up in the same volume of water.

For amino acid determinations, aliquots were diluted to 0.5 ml with water, 0.5 ml ninhydrin reagent (Moore and Stein, 1948) was added, and the reaction was heated at 100°C for 20 min. Samples were cooled rapidly, before dilution with 2.5 ml of 50% ethanol. The optical densities of samples were measured at 570 nm. Leucine was used as the standard.

For iron determinations, aliquots were removed from the HCl hydrolysates, diluted to 2 ml with water, and mixed with 1 ml of freshly prepared buffered tripyridyl-*s*-triazine (Fischer and Price, 1964). The optical density of the colored iron complex was measured at 593 nm. Ferrous ammonium sulfate and ferric chloride were used as standards.

#### *Preparation of <sup>59</sup>Fe-Labeled Spph's and Spph Homogenates*

Heat-shocked spores were spread on GA plates with <sup>59</sup>FeCl<sub>3</sub> at a given specific activity. Spph's were harvested from the plates after 4–5 days and ground in a mortar with several volumes of a cold, buffered isotonic sucrose solution (0.44 M sucrose, 0.05 M phosphate buffer, pH 6.7). The homogenate was filtered through Miracloth (Johnson & Johnson Filter Products Div., Chicago, Ill.) to remove spph cell walls and large debris prior to fractionation by differential centrifugation as described in the text.

Spore homogenates were prepared by mixing spore suspensions in a ratio of 1:2 with 2 mm glass beads, and shaking the slurry in the stainless steel capsule of the Nossal Cell Disintegrator (Nossal, 1953) for 1½–2 min.

#### *Sucrose Gradients*

Linear sucrose gradients (5 ml, 5–20%) were performed in cellulose nitrate centrifuge tubes. The gradients were buffered with 0.1 M phosphate at pH 6.7, and contained 0.1 M NaCl. Following centrifugation, drops were collected from the bottom of the centrifuge tube by using a canula piercing unit

(Buchler Instruments, Inc., Fort Lee, N. J.). The RNA bacteriophage MS2 (81S) was used as a sedimentation marker. Assay of MS2 followed the procedure of Strauss and Sinsheimer (1963).

### *Radioactivity Measurement*

Aqueous samples (1 ml) were added to 10 ml portions of Bray's scintillation solution (Bray, 1960) in glass or polyethylene vials, and their radioactivity was measured with a Packard Tri-Carb scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

## RESULTS

### PART I

#### *Purification and Properties of Phycomyces Ferritin*

Ferritin was usually isolated from mycelium or spph's of *Phycomyces* grown on iron-enriched GA medium. Spores were also found to be a good source of starting material (see Part II), although homogenization of spores was possible only on a small scale. Typically, 100–300 g wet weight of tissue was homogenized in several volumes of cold 0.5 M phosphate buffer (pH 6.0) in a Waring Blendor. This suspension was cycled twice through a Gaulin homogenizer to break the mycelium.

Since *Phycomyces* ferritin is associated with lipids in the cell (see Part II), the initial fractionation of the crude homogenate involved extraction with *n*-butanol (Morton, 1953). This step was particularly useful since most cellular proteins precipitated at the interface between the butanol phase, containing the lipid, and the aqueous phase, containing the ferritin. Effective butanol extraction required a high salt concentration (0.5 M) in the aqueous phase, and an elevated temperature (35–40°C).

To 1500 ml of crude homogenate at 40°C, 600 ml of *n*-butanol was added with vigorous stirring over a 10 min period. The resultant emulsion was separated into two phases by centrifugation at 10,000 *g* for 10 min. The aqueous phase was dialyzed against 0.05 M phosphate buffer to remove salt and butanol, and was concentrated by lyophilization.

The lyophilized material was resuspended in a 10-fold smaller volume, and dialyzed against 0.1 M acetate buffer, pH 5.0. At this pH, *Phycomyces* ferritin was selectively precipitated. The precipitated ferritin was collected by low-speed

centrifugation and dissolved in 0.5 M phosphate buffer, pH 6.0. Ferritin molecules having high iron contents were selected by equilibrium sedimentation in CsCl density gradients.

Apo-ferritin was prepared from pH 5-precipitated ferritin by reduction and dialysis in 2%  $\text{Na}_2\text{S}_2\text{O}_4$  in 0.1 M sodium acetate buffer, pH 5 (Behrens and Taubert, 1952). *Phycomyces* apo-ferritin precipitated slowly during this procedure but redissolved readily upon dialysis in 0.2 M sodium phosphate buffer, pH 7.5. Such apo-ferritin preparations were free of iron and had a typical protein absorption spectrum with a sharp maximum at 280 nm.

### Acrylamide Gel Electrophoresis

The purity of *Phycomyces* ferritin was assessed by: (a) electrophoresis of native ferritin in 5% gels at pH 7.5, and (b) electrophoresis of the ferritin subunit-SDS complex following dissociation of native ferritin with 1% SDS and 1% mercaptoethanol.

Electrophoresis of *Phycomyces* ferritin precipitated at pH 5 yielded one principal component, a minor dimer component (Harrison and Gregory, 1965), and some aggregated material which did not enter the gel (Fig. 1 a). All components contained both iron and protein. Dissociation of ferritin with SDS and electrophoresis of the subunit-SDS complex yielded only one component (Fig. 1 b). On the basis of these results, we conclude that *Phycomyces* ferritin prepared by *n*-butanol extraction and pH 5 precipitation is probably free of contaminating protein, despite some aggregation.

SDS-acrylamide gel electrophoresis was also used to estimate the molecular weight of the ferritin subunit (Weber and Osborn, 1969). Myoglobin (mol wt 17,200), horse ferritin (subunit mol wt 24,000), and ovalbumin (mol wt 43,000) were used as markers of known molecular weight. Interpolating from these markers, we determined that the molecular weight of the subunit of *Phycomyces* ferritin was 25,000, similar to the molecular weight of the horse ferritin subunit.

### UV Absorption Spectrum; Protein and Iron Content

Concentrated solutions of mammalian ferritin are red because of the ferric iron in the core of the molecule. The color is due to continuously

increasing absorption, starting in the blue and running into the UV (Drysdale and Munro, 1965). A similar spectrum was found for *Phycomyces* ferritin, from 240 to 450 nm (Fig. 2). At the longer wavelengths, mammalian ferritin and *Phycomyces* ferritin have identical spectra, reflecting the presence of ferric iron in both. Below 300 nm the absorbance of *Phycomyces* ferritin becomes slightly higher. This increase in absorbance is associated with the higher amount of protein, relative to iron, in *Phycomyces* ferritin.

Determination of the protein and iron content of *Phycomyces* ferritin was done on hydrolysates by using the ninhydrin reagent to determine amino acids, and tripyridyl-*s*-triazine to deter-

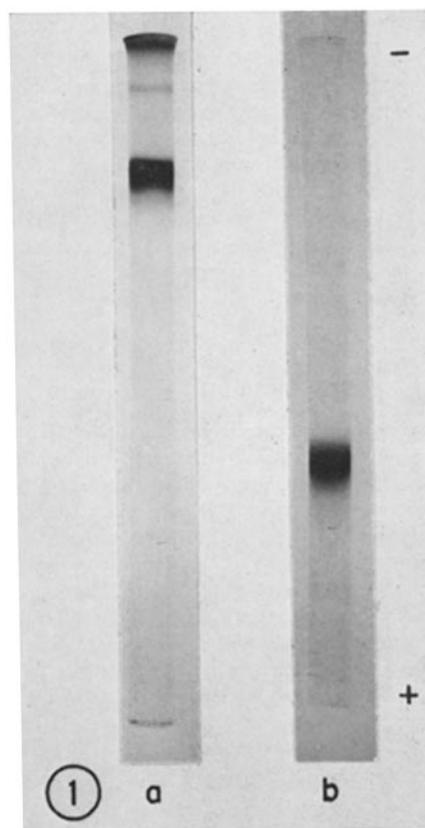


FIGURE 1 Acrylamide gel electrophoresis of native ferritin (a) and ferritin subunit-SDS complex (b). For details see Materials and Methods. Migration was towards the anode. Bromphenol blue (used to mark the front) migrated out of the gel in (a), and just to the end of the gel in (b). Shorter runs of the type shown in (a) revealed no fast-moving proteins.

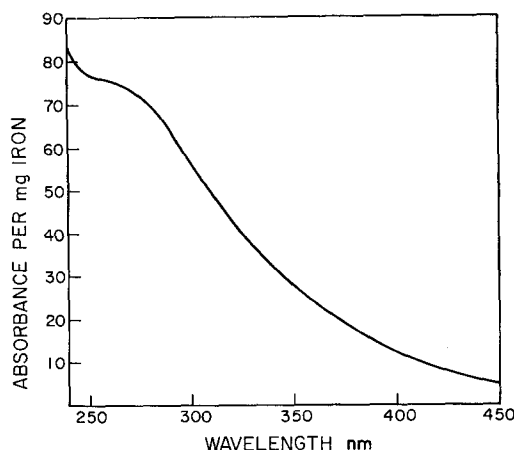


FIGURE 2 UV absorption spectrum of *Phycomyces* ferritin. Absorbance is plotted per milligram Fe as determined from the iron assay. The absorbance due to apoferritin on this scale would be approximately 5 at 280 nm. Absorbance measurements were made in a Cary 15 recording spectrophotometer (Cary Instruments, Monrovia, Calif.) with a 1 cm pathlength.

mine iron. Gamma globulin and mixtures of iron and gamma globulin were hydrolyzed and assayed as controls for recovery of amino acids and iron. Horse ferritin samples were also hydrolyzed as controls for ferritin of known iron content. The results of several independent hydrolysates are given in Table I.

Selection of the highest density fractions from CsCl gradients yielded *Phycomyces* ferritin having P/Fe ratios of about 5. Similar high-density fractions of horse ferritin yielded the expected P/Fe ratio of about 3 (Fischbach and Anderegg, 1965). Total *Phycomyces* ferritin (pH 5 precipitated) had an average P/Fe ratio of 10–11, which is about two times higher than that of total horse ferritin preparations. Thus, both maximally and on the average, *Phycomyces* ferritin contains about one-half as much Fe per molecule as horse ferritin.

#### Determination of Sedimentation Coefficient and Buoyant Density in CsCl

The sedimentation coefficients of ferritin and apoferritin were determined by boundary velocity sedimentation (Schachman, 1959). The measured sedimentation coefficients are given in Table II. Since the sedimentation runs were done at low protein concentration in dilute aqueous buffer, the observed S values are probably quite close to

$S_{20, w}$ . The results are very similar to S values for horse apoferritin (17.6S; Rothen, 1944) and horse ferritin having a P/Fe ratio of 5 (51S; Fischbach and Anderegg, 1965).

The buoyant density of *Phycomyces* ferritin in CsCl was determined by centrifuging solutions of ferritin in CsCl ( $\rho = 1.81 \text{ g/cm}^3$ ) for 36 hr at 44,770 rpm. The buoyant density of the ferritin band formed at equilibrium was determined from the calculated composition density gradient as described by Vinograd and Hearst (1962). Ferritin from *Phycomyces* grown on iron-supplemented medium had a buoyant density of  $1.82 \text{ g/cm}^3$ . It formed a relatively narrow band (a band width of 0.38 cm is equivalent to a density difference of  $0.046 \text{ g/cm}^3$ ), indicating rather less density heterogeneity than in similar mammalian preparations.

#### Electron Microscopy

The morphology of *Phycomyces* ferritin was examined in the electron microscope in the unstained condition and after negative staining (Fig. 3). Its appearance was the same as that of horse ferritin: an electron-opaque core 50–60 Å in diameter which could be seen, in the negatively stained preparation, to be surrounded by an electron-lucent annulus with an over-all diameter of 105 Å.

#### Electron Diffraction

The structure of the iron cores of *Phycomyces* ferritin and horse ferritin were compared by electron diffraction (Harrison et al., 1967). The three strongest rings observed in the horse ferritin pattern (2.52 Å, 2.23 Å, and 1.48 Å) were the only ones clearly visible in the *Phycomyces* pattern. Of the other (weaker) lines present in the horse ferritin pattern (1.95 Å, 1.74 Å, 1.29 Å, 1.12 Å, 1.06 Å, 0.88 Å, and 0.83 Å), only one at 1.95 Å was possibly present in the *Phycomyces* pattern. The three rings present in the *Phycomyces* pattern were qualitatively weaker and broader than those in horse ferritin, despite a greater area density of *Phycomyces* ferritin on the grids. This may only reflect the lower iron content per core in *Phycomyces* ferritin. Nevertheless, the identity of the three lines in the *Phycomyces* pattern with those in the horse pattern cannot be considered sufficient proof that the two core structures are identical.

TABLE I  
*Protein and Iron Content of Phycomyces Ferritin*

Each line represents an independently prepared hydrolysate. Each protein value is the average of four to six ninhydrin assays of the same hydrolysate with the standard deviation from the mean. Iron determinations were in all cases reproducible to within 2%.

Total volume per hydrolysate was 1.1 ml. The assay volume was 0.05 ml. Thus, the recovery of amino acids was essentially complete and was not affected by the presence of iron during hydrolysis. (Losses due to destruction of tryptophan during hydrolysis are too small to be observed in this assay for total amino acids). Iron was quantitatively recovered.

	Protein*	Iron	P/Fe
	$\mu\text{g/assay}$	$\mu\text{g/assay}$	
Phycomyces ferritin			
pH 5 precipitated	22.3 $\pm$ 0.8	2.00	11.1
	20.5 $\pm$ 0.9	2.00	10.3
Main band from CsCl gradient	14.8 $\pm$ 1.2	3.30	4.5
High density side	15.3 $\pm$ 0.9	3.20	4.8
	7.0 $\pm$ 0.5	1.65	4.2
	8.7 $\pm$ 0.2	1.65	5.3
Low density side	16.5 $\pm$ 1.1	2.95	5.6
	16.5 $\pm$ 0.6	3.10	5.3
	10.1 $\pm$ 0.2	1.65	6.1
	8.5 $\pm$ 0.9	1.65	5.2
Controls			
Gamma globulin (450 $\mu\text{g/hydrolysate}$ )	19.5 $\pm$ 0.3	—	—
	20.8 $\pm$ 0.3	—	—
Gamma globulin (450 $\mu\text{g/hydrolysate}$ ) + iron† (50 $\mu\text{g/hydrolysate}$ )	20.0 $\pm$ 0.7	2.30	8.7
	20.1 $\pm$ 0.7	2.30	8.7
Horse ferritin selected from CsCl gradient at density 1.8 g/cm <sup>3</sup>	22.6 $\pm$ 1.2	8.60	2.6
	22.0 $\pm$ 1.5	8.60	2.6

\* Refers to micrograms of amino acid as determined with ninhydrin reagent.

† Added as FeCl<sub>3</sub>.

## RESULTS

### PART II

#### *Intracellular Ferritin: Localization*

*Phycomyces* ferritin is bound to lipid droplets in the cytoplasm of spph's, mycelium, and spores. This ferritin-lipid complex forms an unusual subcellular structure which is clearly visible in electron micrographs of spph's in thin section (Fig. 4). In osmium tetroxide-fixed tissue, the lipid droplets in the spph cytoplasm are large, round, uniformly staining objects 0.5–2.0  $\mu$  in diameter. At high magnification, dense granules

TABLE II  
*Sedimentation Coefficients of Phycomyces Ferritin and Apoferritin*

Boundary sedimentation was performed at 20°C in 0.05 M NaPO<sub>4</sub> buffer, pH 7.5.

	Protein concentration	S <sub>observed</sub>
	$\text{mg/ml}$	
Ferritin	0.04	54.8
	0.08	55.2
Apoferritin	0.80	18.0

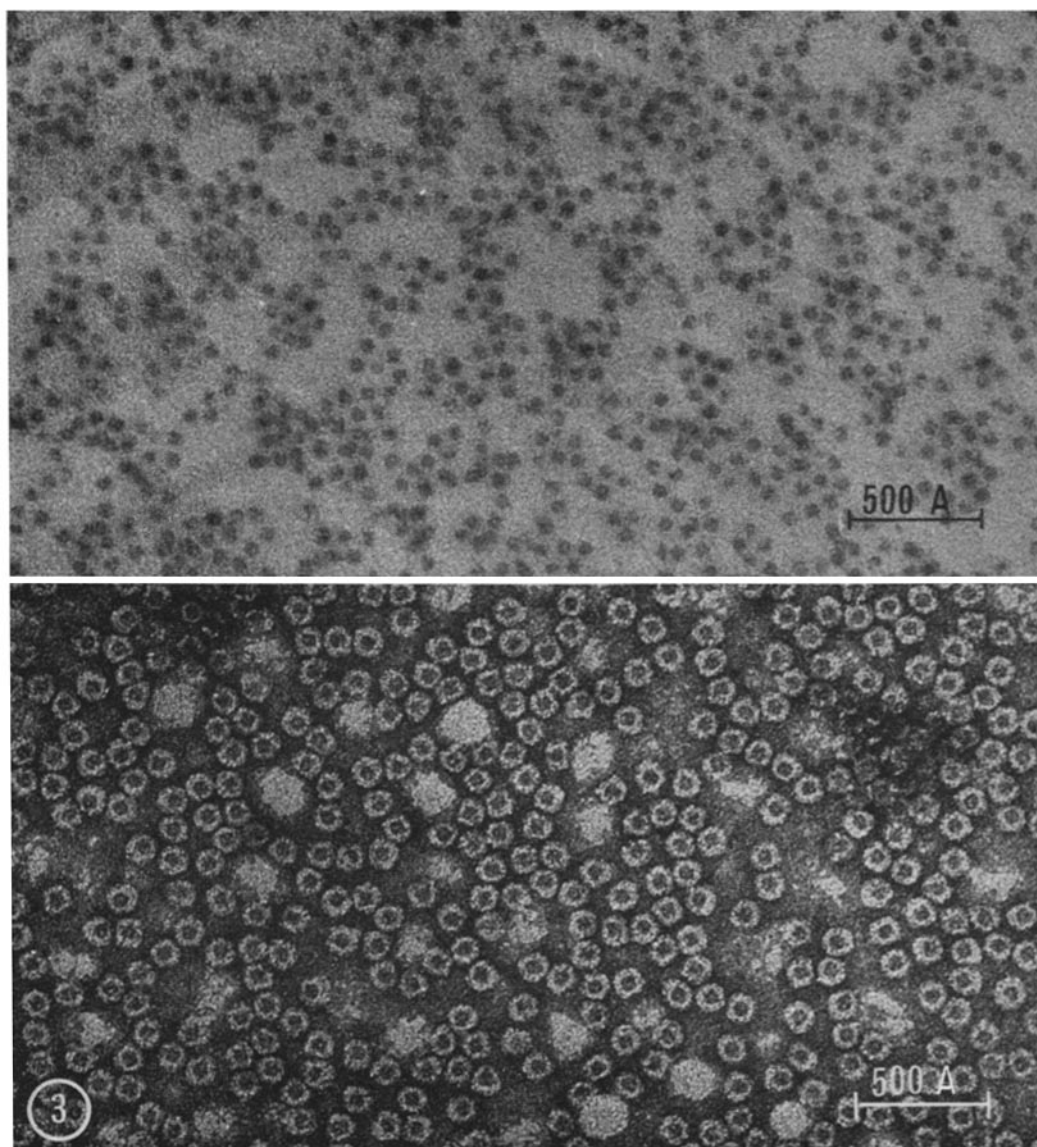


FIGURE 3 *Phycomyces* ferritin. *Top*: unstained. *Bottom*: negatively stained with uranyl acetate.  $\times$  340,000.

are visible around the edge or over part of the surface of each lipid droplet, depending on whether the section through the droplet is equatorial or tangential. These granules are arranged in a partially crystalline, two-dimensional pattern over the surface of the lipid droplet. They have the same dimensions as *Phycomyces* ferritin described in Part I, and are electron-opaque when seen without heavy metal fixative or stain.

A simpler, more effective method of examining the ferritin-lipid complex was developed with the use of sph homogenates prepared in buffered isotonic sucrose solutions. When such homogenates were centrifuged (10,000 *g* for 10 min), the lipid droplets sedimented centripetally because of their low density, and collected in a thick pellicle on top of the solution. Dilution of the homogenate prior to centrifugation produced a monolayer of

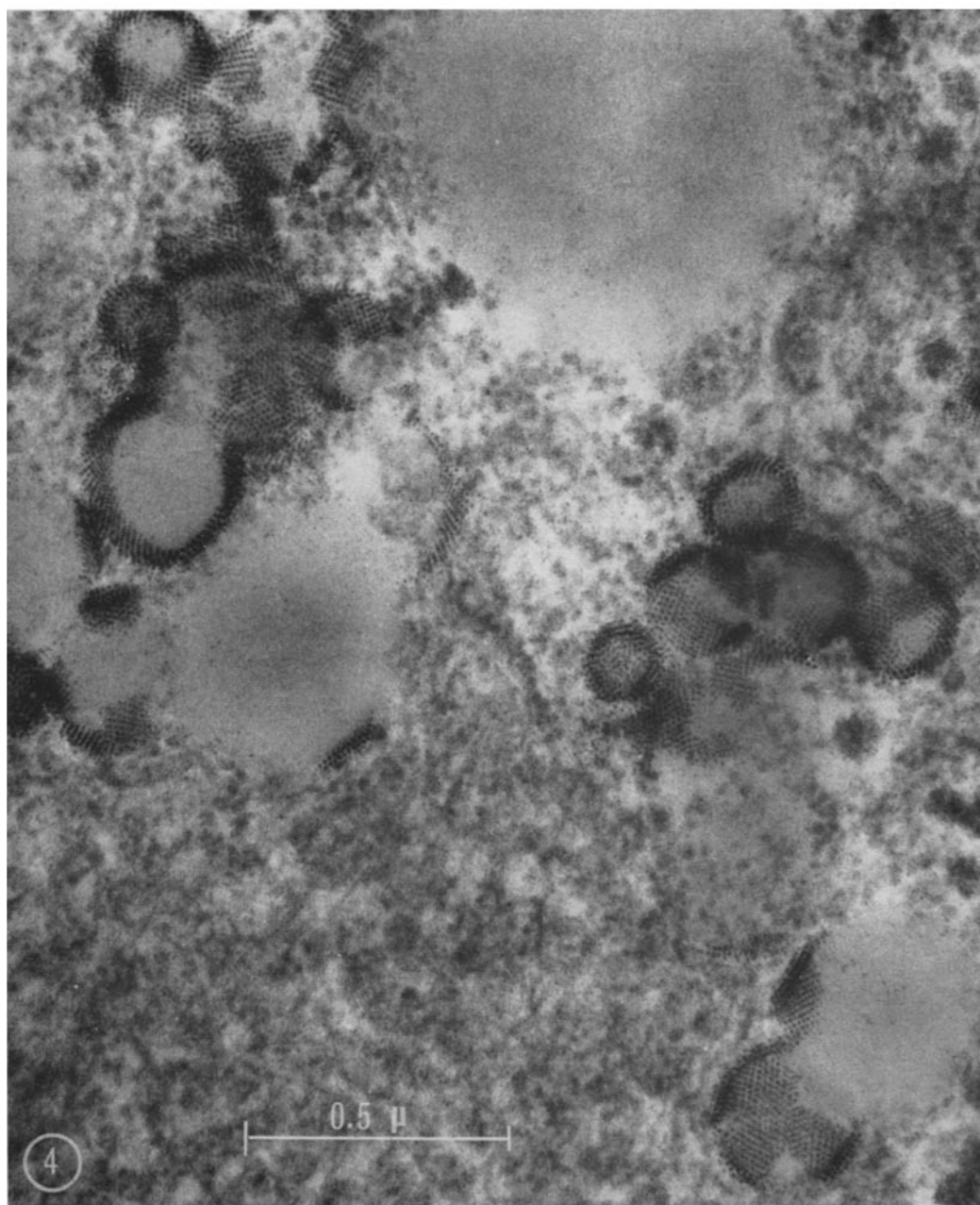


FIGURE 4 Thin section near the top of a glutaraldehyde-osmium tetroxide-fixed stage III spph showing lipid droplets covered with crystalline arrays of ferritin. Some of the lipid droplets are cut tangentially showing extensive crystalline regions; others are cut more equatorially showing only a ring of ferritin. The medium was not supplemented with iron.  $\times 83,000$ .



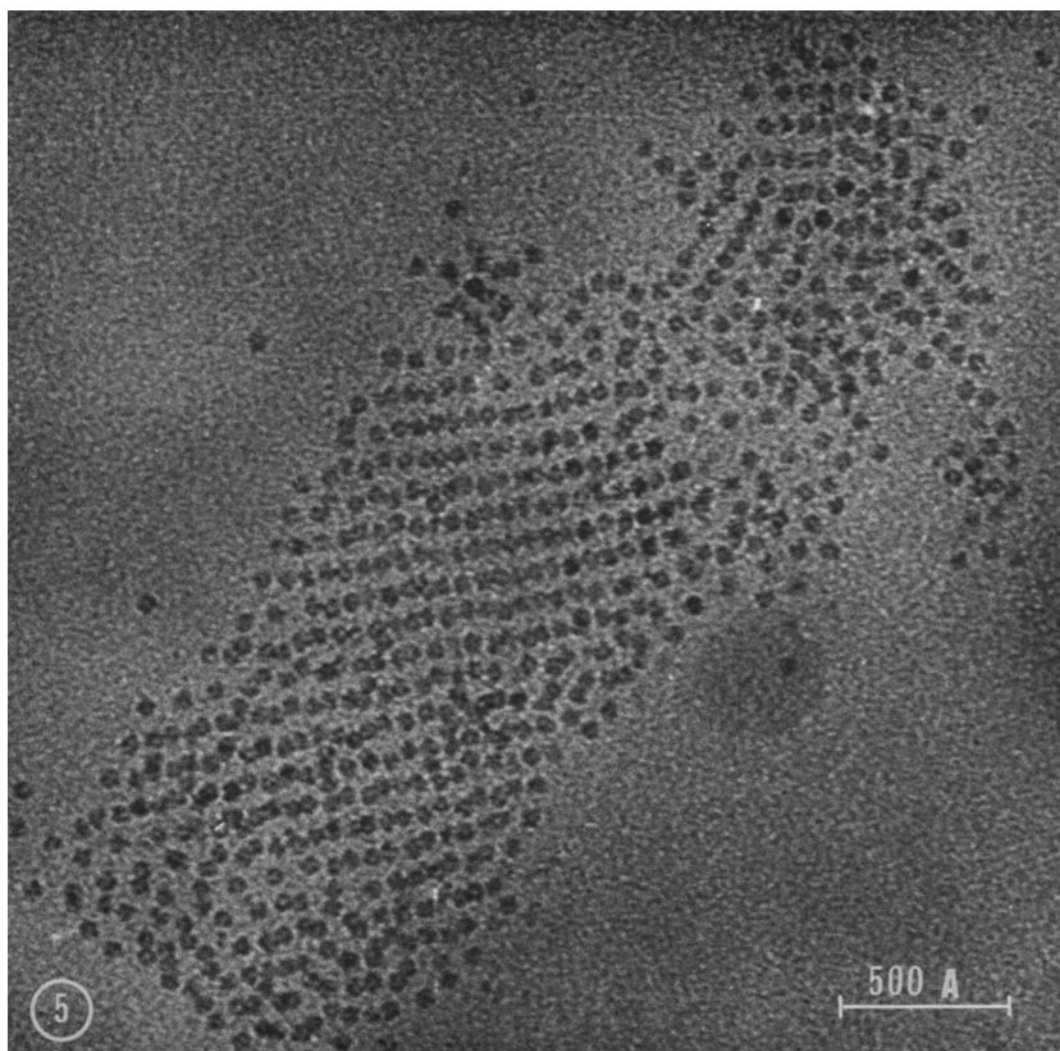


FIGURE 5 Crystalline monolayer of *Phycomyces* ferritin from the surface of a lipid droplet. The preparation was obtained by diluting a spph homogenate (from iron-supplemented medium) to the point where centrifugation produced a monolayer of lipid on the surface of the solution. A specimen grid was touched to the surface of the solution, washed with a drop of water, dried, and examined without staining in the electron microscope. The electron-opaque cores of ferritin are visible.  $\times 444,000$ .

lipid droplets instead of a pellicle. Such monolayers could be picked up on electron microscope grids, washed with several drops of water to remove most of the lipid, and examined directly in the electron microscope (Fig. 5). In such preparations the crystalline array of ferritin molecules from the surface of the lipid droplets stuck to the grid. At high magnification the fine structure typical of ferritin was easily resolved.

#### *Distribution of Ferritin among Subcellular Fractions of *Phycomyces* Spph's*

To determine the distribution of ferritin in spph's, *Phycomyces* was grown on  $^{59}\text{Fe}$ -supplemented medium, spph's were harvested, and the resulting homogenate was fractionated by differential centrifugation. Low-speed centrifugation (500 g) pelleted the spores, nuclei, and aggre-

TABLE III

*Distribution of  $^{59}\text{Fe}$  and Ferritin in Spph Homogenate*

*Phycomyces* was grown on GA medium supplemented with 500  $\mu\text{g}$  of Fe per Petri dish.  $^{59}\text{FeCl}_3$  was added to a specific activity  $4.2 \times 10^5$  cpm/mg Fe. Spph's were harvested from 20 Petri dishes and ground in buffered isotonic sucrose in a mortar.

Fraction	Total cpm $\times 10^{-5}$	%	Amount of $^{59}\text{Fe}$ in ferritin (as determined by sedimentation in 55S region of sucrose gradient)
Homogenate	4.7	100	—
500 g pellet (spores)	3.5	72	100% of extractable $^{59}\text{Fe}$ (but only ~50% of total cpm are extractable)
10,000 g pellicle (lipid droplets)	0.4	8	100%
10,000 g supernatant	0.8	16	Variable but <50%
10,000 g pellet (mitochondria, glycogen)	0.2	4	Not investigated

gated debris. Centrifugation of the low-speed supernatant for 15 min at 10,000 g pelleted the mitochondria and glycogen, and collected the lipid droplets on top of the solution (pellicle). Table III gives the distribution of  $^{59}\text{Fe}$  among these fractions. The bulk of the  $^{59}\text{Fe}$  was incorporated into the low-speed spore pellet. Subsequent purification of the spores from the nuclei and debris in this fraction (by centrifugation through 1.9 M sucrose and repeated washings in fresh buffer) caused no loss of  $^{59}\text{Fe}$ , indicating that the label was located in the spores.

The amount of  $^{59}\text{Fe}$  in ferritin in each subcellular fraction was determined by sedimenting samples in sucrose gradients. Prior to sedimentation, ferritin was freed from any associated lipid in the fractions by treatment with *n*-butanol (see Part I). Spores were homogenized in a Nossal cell disintegrator (Nossal, 1953) before *n*-butanol extraction. The results are given in Table III.

The lipid fraction contained only  $^{59}\text{Fe}$ , which sedimented in the 55S region of the gradient and was therefore identified as ferritin (Fig. 6). In a spore homogenate, all the extractable radioactivity sedimented at about 55S in a sucrose gradient, similar to the result obtained with the lipid fraction (Fig. 7). It should be noted, however, that only 40–50% of the  $^{59}\text{Fe}$  in a spore homogenate could be recovered in the aqueous phase after butanol extraction. Re-extraction of the precipitated material at the interface, containing the remaining radioactive matter, yielded some more  $^{59}\text{Fe}$  which sedimented as ferritin, although it was never possible to recover all of the  $^{59}\text{Fe}$

from spore homogenates. No radioactive matter which sedimented differently than ferritin was recovered.

The nature of the  $^{59}\text{Fe}$  in the 10,000 g supernatant varied from experiment to experiment. At least 50% of the radioactive matter was always dialyzable; of the remaining  $^{59}\text{Fe}$ , some always sedimented as ferritin. We believe this ferritin to be molecules released from ferritin-lipid complexes during homogenization, rather than ferritin naturally free in the cytoplasm, since such free ferritin is not seen in electron micrographs of spph's.

#### *Ferritin Content of Spph's and Spores as a Function of Iron Content of the Growth Medium*

Much of the work already discussed in this paper has depended on the fact that supplemental iron in the growth medium markedly enhances the ferritin content of *Phycomyces*. This effect has been examined quantitatively. GA medium containing two levels of iron was used: iron poor (no iron added to GA medium; iron level in the medium due to impurities is 0.1  $\mu\text{g}/\text{ml}$ ) and iron rich (GA medium supplemented with 15  $\mu\text{g}/\text{ml}$  of Fe). *Phycomyces* was grown at these two iron levels in the presence of  $^{59}\text{FeCl}_3$ . Spph homogenates (from 30 Petri dishes; approximately 30 g total wet weight) were prepared and separated by low-speed centrifugation into a spore fraction and a cytoplasmic fraction. (The cytoplasmic fraction contained the mitochondria, glycogen, lipid, and supernatant fractions which

were examined independently in the previous section.) The distribution of  $^{59}\text{Fe}$  between these two fractions is given in Table IV. The absolute amounts of iron calculated from the specific activities are also given in the table. It is evident that the iron content, both of the spph cytoplasm and of the spores, increases sharply when the growth medium is supplemented with iron.

The cytoplasmic and spore fractions were extracted for ferritin analysis by using the *n*-butanol procedure. The cytoplasmic fraction was treated directly with *n*-butanol. The spores were first homogenized in a Nossal cell disintegrator, and then extracted with *n*-butanol. Between one-third and two-thirds of the  $^{59}\text{Fe}$  in cytoplasmic and spore homogenates of both iron-rich and iron-poor samples was recovered in the aqueous

phase following butanol extraction. The aqueous phases from these extractions were analyzed for ferritin by centrifuging samples in sucrose gradients. The results are given in Fig. 7. All extractable radioactivity from both iron-rich and iron-poor spores sedimented in the 55S region of the gradients. In the two cytoplasmic samples, about half of the  $^{59}\text{Fe}$  also sedimented as ferritin. The conclusion from this experiment is that the additional iron in spores and spph cytoplasm from iron-rich growth medium is contained largely in ferritin.

#### *Sedimentation Properties of Cytoplasmic and Spore Ferritin from Iron-Rich and Iron-Poor Growth Media*

Supplementing the growth medium with iron not only induces the formation of additional ferritin in both cytoplasm and spores, but also alters the sedimentation properties (i.e. iron content) of the average ferritin molecule. This is shown in the four sucrose gradients in Fig. 7. When the growth medium was supplemented with iron, the ferritin isolated from spores and spph cytoplasm was indistinguishable by sedimentation. It had a value of 55S. By comparison, on iron-poor medium, the ferritin from spores was relatively homogeneous and sedimented at 45S, whereas the ferritin from spph cytoplasm was very heterogeneous and sedimented over a broad range of values from 55 to 30S. Clearly, under iron-poor growth conditions, two populations of ferritin exist in *Phycomyces* spph: one in spph cytoplasm and one in spores.

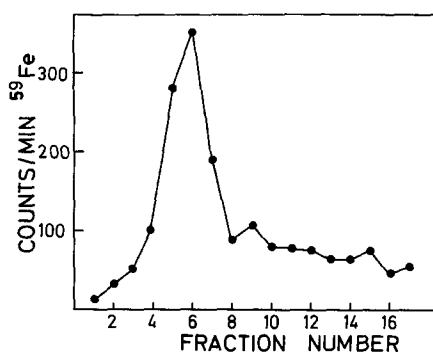


FIGURE 6 Sucrose gradient sedimentation of  $^{59}\text{Fe}$ -labeled lipid pellicle treated with *n*-butanol to dissociate the ferritin from the lipid droplets. The material was centrifuged at 37,000 rpm for 2 hr at 4°C. Sedimentation is from right to left.

TABLE IV

#### *$^{59}\text{Fe}$ Content of Spph Cytoplasm and Spores from Iron-Poor and Iron-Rich Growth Media*

Approximately 30 g wet weight of *Phycomyces* spph's were used in each homogenate. The same number of spores was recovered from the iron-rich and iron-poor homogenates.

Fraction	Iron rich			Iron poor		
	$\text{cpm} \times 10^{-6}$	$\mu\text{g Fe}$	%	$\text{cpm} \times 10^{-6}$	$\mu\text{g Fe}$	%
Homogenate	14	1900	100	52.7	48	100
500 g supernatant (cytoplasm)	4.7	640	35	10.2	9.2	22
500 g pellet (spores)	10	1360	65	41.4	38	78
Specific activity:						
Iron-rich medium				$0.74 \times 10^7 \text{ cpm/mg}$		
Iron-poor medium				$1.1 \times 10^9 \text{ cpm/mg}$		

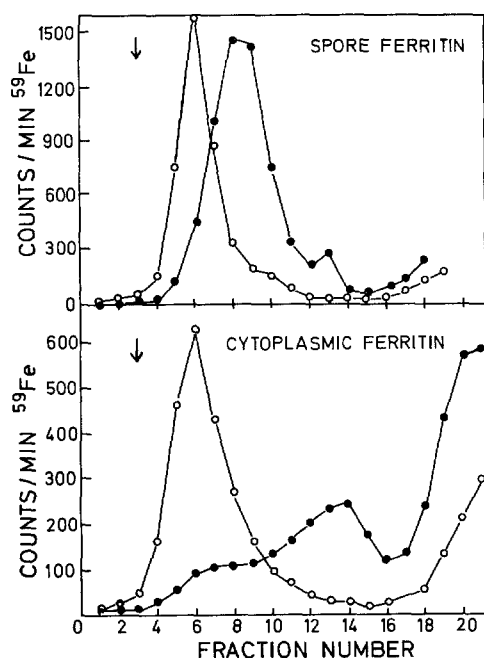


FIGURE 7 Sedimentation properties of cytoplasmic ferritin and spore ferritin. Homogenates of *sph*'s grown on iron-rich and iron-poor media (containing  $^{59}\text{Fe}$ ) were separated into spore and cytoplasmic fractions. Samples from the aqueous phase of *n*-butanol-extracted fractions were layered on sucrose gradients and centrifuged for 2 hr at 37,000 rpm, 4°C. 20-drop fractions were collected for  $^{59}\text{Fe}$  assay. One drop between each fraction was collected into broth for assay of bacteriophage MS2 added before centrifugation to each sample as a sedimentation marker at 81S. To facilitate comparison, the results from the iron-rich and iron-poor samples have been superimposed. The position of the MS2 peak is indicated by the arrow. Sedimentation is from right to left. o—o, iron rich; ●—●, iron poor.

## DISCUSSION

Part I of this paper presents the purification procedure for a large, iron-containing protein from *Phycomyces* having properties similar to those of mammalian ferritins. On the basis of these similarities, we have identified it as *Phycomyces* ferritin. The purification procedure yields a single molecular species, as judged by acrylamide gel electrophoresis, which can be shown in electron micrographs to consist of a spherical protein shell surrounding an iron-containing core. Reduction with  $\text{Na}_2\text{S}_2\text{O}_4$  and dialysis of *Phycomyces* ferritin removes the iron core, leaving only the protein

shell (apoferritin). *Phycomyces* apoferritin is similar in size (18S) to mammalian apoferritin, and also consists of similar subunits (each of mol wt 25,000).

Although similar in most respects, there are several indications that *Phycomyces* ferritin contains maximally only one-half as much iron per molecule as mammalian ferritin. Total *Phycomyces* ferritin (from iron-supplemented medium) has a protein-to-iron (P/Fe) ratio of 10–11, compared to about 5 for total horse ferritin; selected high-density fractions of *Phycomyces* ferritin have P/Fe ratios of 5, compared to 2.5 for high-density horse ferritin fractions. Since the protein moieties of both ferritins appear to have the same molecular weight (sedimentation coefficient), the 2-fold difference in P/Fe ratio must be due to a 2-fold lower iron content per molecule in *Phycomyces* ferritin. This decreased iron content per molecule is also reflected in the sedimentation coefficient—55S—of *Phycomyces* ferritin. Horse ferritin molecules having P/Fe ratios of 5 (i.e. about one-half the full complement of iron) sediment at 51S (Fischbach and Anderegg, 1965). The buoyant density of *Phycomyces* ferritin, however, does not agree well with the lower iron content per molecule. The expected density for ferritin with a P/Fe ratio of 5 would be about 1.65 g/cm<sup>3</sup>, rather than the observed 1.82 g/cm<sup>3</sup> (Fischbach and Anderegg, 1965). We can offer no explanation at present for this surprising difference. The possibility that the *Phycomyces* ferritin core has a somewhat different structure, and thus a different density, has not been excluded by our electron diffraction results.

The localization of *Phycomyces* ferritin in crystalline monolayers on the surface of lipid droplets is unique. By comparison, mammalian ferritin occurs scattered throughout the cytoplasm as individual molecules (Kuff and Dalton, 1957; Kerr and Muir, 1960; Bessis and Breton-Gorius, 1959), and plant ferritin, although found in monolayers in proplastids, does not appear to be associated with lipid droplets (Hyde et al., 1963; Robards and Humpherson, 1967). The exact structure of the ferritin-lipid complex in *Phycomyces* is still unclear. The lipid pellicle produced by centrifugation of *sph* homogenates consists principally of triglycerides, ergosterol, and  $\beta$ -carotene, but also contains some protein (Stoffel, personal communication). Thus, the possibility exists of a membrane around the lipid droplets to which

ferritin is bound. Neither we nor Zalokar (1969) have been able to identify such a membrane in electron micrographs, although Zalokar has suggested that it may exist because ferritin monolayers detached from lipid droplets can be found in disrupted cytoplasm.

This paper has demonstrated a 50-fold increase in the ferritin content of *Phycomyces* when the fungus is grown on iron-supplemented medium. Similar increases in ferritin content following iron administration occur in mammalian systems, and are associated with an increase in the net synthesis of the protein moiety of ferritin (Fineberg and Greenberg, 1955; Loftfield and Eigner, 1958; Saddi and von der Decken, 1965; and Drysdale and Munro, 1966). The magnitude of the ferritin increase in *Phycomyces* strongly suggests that *de novo* apoferritin synthesis has been induced by the presence of supplemental iron in the growth medium. No direct evidence, however, has been obtained.

In conclusion, mention should be made of the advantage of *Phycomyces* for studying the role of ferritin in iron metabolism. Despite extensive study in mammalian systems, little is known about the mechanisms and control of iron incorporation into, and release from, ferritin. This is the result of the fact that iron metabolism in mammals is a complicated process involving several organs (bone marrow, liver, spleen, and blood), and several molecular intermediates (transferrin, ferritin, and hemoglobin), none of which can be easily isolated from the rest for the purpose of investigation. By comparison, *Phycomyces* spores offer a simpler system. The results of this paper show that ferritin is selectively incorporated into spores (Tables III and IV), where it probably functions as a storage form of iron for biosynthetic steps requiring iron during early stages of spore development. The results also show that the amount of ferritin per spore can be regulated. Thus, germinating spores of *Phycomyces* offer a system in which intracellular ferritin concentrations and extracellular iron concentrations can be experimentally manipulated to study ferritin iron metabolism. *Phycomyces* also offers to the investigator the possibility of selecting mutants with defective or altered iron metabolism (Bergman et al., 1969).

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